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(54) Title: SCREEN FOR NITRIC OXIDE SYNTHASE MODULATORS

(57) Abstract: A method for identifying a modulator of NOS activity comprises: (i) providing a polynucleotide construct which comprises: (a) a promoter operably linked to a coding sequence, wherein the promoter is responsive to ecdysone or an analog thereof and the coding sequence encodes a nitric oxide synthase (NOS) or a functional variant thereof; or (b) a promoter operably linked to one or more tetracycline operator site sequences and a coding sequence in that order, wherein the coding sequence encodes a nitric oxide synthase (NOS) or a functional variant thereof; (ii) contacting a test substance with the construct under conditions that would permit the expression and activity of the NOS encoded by the coding sequence in the absence of the test substance; and (iii) determining thereby whether the said substance modulates NOS activity. Modulators of nitric oxide synthase activity can be used in the treatment of conditions in which the abnormal metabolism of nitric oxide is implicated.

SCREEN FOR NITRIC OXIDE SYNTHASE MODULATORS

Field of the Invention

This invention relates to methods for identifying modulators of nitric oxide synthases. Modulators of nitric oxide synthase activity can be used in the treatment of conditions in which the abnormal metabolism of nitric oxide is implicated.

Background to the Invention

Over-expression and under-expression of the various mammalian isoforms of the enzyme nitric oxide synthase (NOS) have been reported to correlate with various disease conditions. Thus, inhibition of NOS is seen as a starting point for the development of drugs effective against those diseases.

A good source of NOS is therefore required and ideally screening procedures for identifying inhibitors of NOS should involve a high through-put cell-based screen (HTS) against the human isoforms.

A major problem with this approach however is that the constitutive expression of NOS in a recombinant cell line is often toxic to the cell. This leads to decreased levels of NO generation as the cell is maintained and passaged. The alternative possibility, of using the cytokine-mediated generation of the inducible NOS (iNOS), is prohibitively expensive for HTS strategies. Furthermore, the cocktails of cytokines used in such protocols may themselves significantly interfere with iNOS functions.

Summary of the Invention

We have produced human cell lines that can express one of the three known human nitric oxide synthases, inducible nitric oxide synthase (iNOS), neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS), in the presence of the insect steroid hormone ecdysone or an analog thereof. We have also produced a cell line that can express human iNOS in the presence of tetracycline or an analog thereof.

Thus, we have produced cell lines which are capable of producing NO in a

dose- and time-dependent manner by treatment of the cells with ecdysone or an analog thereof or tetracycline or an analog thereof.

These cell lines thus provide sources of human iNOS, nNOS and eNOS that can be used in high through-put cell based screens for the identification of substances which can modulate the activity of those enzymes. Modulators of activity of NOS enzymes are substances that can either activate or inhibit the activity of those enzymes.

According to the present invention there is thus provided a method for identifying a modulator of NOS activity comprising:

(i) providing a polynucleotide construct which comprises:

(a) a promoter operably linked to a coding sequence, wherein the promoter is responsive to ecdysone or an analog thereof and the coding sequence encodes a nitric oxide synthase (NOS) or a functional variant thereof; or

(b) a promoter operably linked to one or more tetracycline operator site sequences and a coding sequence in that order, wherein the coding sequence encodes a nitric oxide synthase (NOS) or a functional variant thereof;

(ii) contacting a test substance with the construct under conditions that would permit the expression and activity of the NOS encoded by the coding sequence in the absence of the test substance; and

(iii) determining thereby whether the said substance modulates NOS activity.

The invention also provides:

- a modulator of NOS activity identified by a method of the invention;
- a modulator of the invention which is an inhibitor of NOS activity for use in a method of prophylaxis or treatment of the human or animal body by therapy;
- use of a modulator of the invention which is an inhibitor of NOS activity for the manufacture of a medicament for use in the prophylaxis or treatment of a clinical condition associated with excessive NO production;
- a pharmaceutical composition comprising a modulator of the invention which

is an inhibitor of NOS activity and a pharmaceutically acceptable carrier or diluent;

- a method of preventing or treating a clinical condition in a host associated with excessive NO production which method comprises administering to the host a therapeutically effective amount of a modulator of the invention which is an inhibitor of NOS activity;
- a modulator of the invention which is an activator of NOS activity for use in a method of prophylaxis or treatment of the human or animal body by therapy;
- use of a modulator of the invention which is an activator of NOS activity for the manufacture of a medicament for use in the treatment of a clinical condition associated with deficient NO production;
- a pharmaceutical composition comprising a modulator of the invention which is an activator of NOS activity and a pharmaceutically acceptable carrier or diluent;
- a method of preventing or treating a host suffering from a clinical condition in a host associated with deficient NO production which method comprises administering to the host a therapeutically effective amount of a modulator of the invention which is an activator of NOS activity;
- a polynucleotide construct comprising:
  - (a) a promoter operably linked to a coding sequence, wherein the promoter is responsive to ecdysone or an analog thereof and the coding sequence encodes a nitric oxide synthase (NOS) or a functional variant thereof; or
  - (b) a promoter operably linked to one or more tetracycline operator site sequences and a coding sequence in that order, wherein the coding sequence encodes a nitric oxide synthase (NOS) or a functional variant thereof;
- a vector which incorporates a polynucleotide construct of the invention; and
- a cell which harbours a polynucleotide construct or vector of the invention.

Brief description of the Drawings

Figure 1(a) shows the plasmid map for pIND-hiNOS-f (human iNOS); Figure 1(b) shows the plasmid map for pIND-hnNOS-f (human nNOS); and Figure 1 (c) shows the plasmid map for p-IND-heNOS-f (human eNOS). Figure 1(d) shows the plasmid map of pTet-hiNOS-f (human iNOS).

Figure 2(a) shows generation of NO by EcR293 clone 11, following treatment with muristerone A. EcR293 clone 11 cells were grown with varying concentrations of muristerone A and at different time intervals supernatants were taken and the Griess reaction was used to measure the nitrite concentration. Figure 2(b) shows Northern and Western blots of carried out on extracts from cells treated with either 1μM or 10μM muristerone A. Extracts were also taken from cells grown in the absence of muristerone A. For Northern blots, filters were probed with a human iNOS cDNA and hybridisation with human β-actin was used as a loading control. For Western blots, filters were probed with a polyclonal antibody raised against the 7 C-terminal residues of human iNOS: Cyc-Arg-Nle-Orn- (Ser-Leu-Glu-Met-Ser-Ala-Leu). The filters were subsequently stripped and re-probed with an anti- human α-tubulin antibody as a loading control.

Figure 3 shows generation of NO in a panel of cell lines transfected with pTet-hiNOS-f. Cells were treated with 1μg/ml tetracycline for 24h and NOS activity was assessed by assaying for accumulated nitrite using the Griess reaction.

Figure 4 shows the results of an assay using two inhibitors of iNOS with EcR293 clone 11 cells. Bars 7 and 8 show control experiments; absence of muristerone A (MuA) or addition 5μM muristerone A (MuA) respectively. Bars 1 to 6 show addition of 5μM muristerone A and serial dilutions of L-^{5}N-iminoethyl-L-ornithine (L-NIO) or 2-amino-5, 6-dihydro-6-methyl-4H-1, 3 thiazine hydrochloride (AMT).

Figure 5 shows the results of an assay using two inhibitors of iNOS with

Tex293 clone 22 cells. Bars 1 and 2 show control experiments; absence of tetracycline (Tet) or addition of 0.025µg/ml tetracycline (Tet) respectively. Bars 3 to 5 show addition of 0.025µg/ml Tet and 20µM L-NIO, 80µM l-NIO or 20µM 1400W respectively.

Detailed Description of the Invention

Constructs. Vectors and Cells

Steroid hormones are small hydrophobic molecules that can diffuse through the plasma membrane of cells where they can bind reversibly to specific steroid-hormone-receptor proteins in the cytoplasm or nucleus. The binding of hormone activates the receptor, enabling it to bind with high affinity to specific DNA sequences that act as transcriptional enhancers. This binding increases the level of transcription from certain nearby genes.

A pulse of the insect steroid hormone ecdysone triggers metamorphosis in *Drosophila melanogaster*, showing effects such as chromosomal puffing within minutes of hormone addition. Mediating this response is the functional ecdysone receptor, which is a heterodimer of the ecdysone receptor (EcR) and the product of the ultraspiracle gene (USP).

Insect hormone responsiveness can be recreated in cultured mammalian cells by cotransfection of a cell with a functional ecdysone receptor (a heterodimer of EcR and USP) and an ecdysone responsive construct and treatment of the cell with ecdysone or an analog thereof.

A tetracycline responsive system can be created in cultured mammalian cells by cotransfection of a cell with a plasmid encoding a tetracycline repressor protein (tetR) and a plasmid containing a tetracycline responsive element linked to a promoter. The promoter sequence is used to drive heterologous gene expression. The tetracycline responsive element comprises particular DNA sequences called tetracycline operator sites, which can bind a homodimer of tetR. If those sequences are positioned between a promoter and a coding sequence in a construct, the presence of tetR bound to a tetracycline operator site will prevent the promoter driving

expression of the coding sequence. However, when tetracycline is added to cells the tetracycline binds to tetR homodimers leading to a conformational change in tetR, such that it is unable to bind a tetracycline operator site. The tetR:tetracycline complex dissociates from the Tet operator site and allows the promoter to drive expression of the coding sequence.

(i) Constructs

The invention provides polynucleotide constructs which are responsive to ecdysone or an analog thereof. The invention also provides polynucleotide constructs which are responsive to tetracycline or an analog thereof.

The ecdysonse responsive constructs comprise a promoter operably linked to a coding sequence, wherein the promoter is responsive to ecdysone or an analog thereof and the coding sequence codes for a NOS or a functional variant or fragment thereof.

The tetracycline responsive constructs comprise a promoter operably linked to one or more teracycline operator site sequences and a coding sequence in that order, wherein the coding sequence codes for a NOS or a functional variant or fragment thereof.

The constructs may comprise DNA or RNA. They may also include within them synthetic or modified nucleotides. A number of different types of modification to polynucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the invention, it is to be understood that the constructs described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or lifespan of constructs of the invention. Constructs of the invention may be produced recombinantly, synthetically, or by any means available to those of skill in the art.

The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. Thus, a regulatory sequence, such as a promoter, "operably linked" to a coding sequence is positioned in such a way that expression of the coding sequence is

achieved under conditions compatible with the regulatory sequence.

A promoter for use in an ecdysone-reponsive construct of the invention may be any promoter which can drive the transcription of a coding sequence to which it is operably linked in the presence of the steroid hormone ecdysone or an analog thereof.

The promoter may be a naturally occurring promoter from a *Drosophila melanogaster* or other insect ecdysone-responsive gene. Alternatively, the promoter may be a non-naturally occuring promoter. A non-naturally occuring promoter may be used which comprises a minimal promoter and an ecdysone-responsive element (EcRE). An EcRE is a nucleotide sequence to which a functional ecdysone receptor can bind in the presence of ecdysone. Suitable minimal promoters include the minimal heat shock promoter.

An ecdysone-responsive promoter may comprise more than one EcRE, for example 2 to10 elements or more preferably 4 to 6 elements. The sequence of an EcRE will depend on the exact functional ecdysone receptor used. If a modified functional ecdysone receptor is used (see below) it may be appropriate to use a modified EcRE (see No *et al.*, *Proc. Natl. Acad. Sci. USA*, **93**: 3346-3351). The EcRE(s) and minimal promoter sequences do not have to be immediately adjacent. Because EcREs function as transcriptional enhancers, they can be placed some distance upstream, for example from 1, 10 or 25 nucleotides to 30, 40, 50, 100, 500 or 1000kb upstream of a minimal promoter. EcREs could even be placed further than 1kb upstream of a minimal promoter. Generally, if multiple copes of an EcRE are used, the mutiple copies will be arranged in an array, one after the other.

Constructs of the invention may be responsive to ecdysone [(*2β, 3β, 5β, 22R*)-2,3,14,22,25-pentahydroxycholest-7-en-6-one] or an analog thereof. Suitable analogs of ecdysone for use in the invention include muristerone A [2β, 3β ,5α, 11α, 14, 20, 22-heptahydroxy-5β, 7-cholesten-6-one] or ponasterone A [(*2β, 3β, 5β, 22R*)-2, 3, 14, 20, 22, 25-pentahydroxycholest-7-en-6-one] and GS™-E (Invitrogen, San Diego, CA; see also Dhadialla *et al.*, 1998, Ann. Rev. Entomol. **43**: 545-569).

A promoter for use in an tetracycline-reponsive construct of the invention may be any promoter which can drive the transcription of a coding sequence to which it is operably linked in the presence of the antibiotic tetracycline or an analog

thereof.

Generally, the choice of promoter will depend on the host cell to be used for expression of the coding sequence. Typically, expression in mammalian cells, for example human cells will be required and thus a mammlian promoter will be preferred. Mammalian promoters, such as β-actin promoters, may be used. Tissue-specific promoters may be used. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR), the rous sarcoma virus (RSV) LTR promoter, the SV40 promoter, the human cytomegalovirus (CMV) IE promoter, adenovirus, HSV promoters (such as the HSV IE promoters), or HPV promoters, particularly the HPV upstream regulatory region (URR). Viral promoters are readily available in the art. Constitutive promoters, for example the CMV promoter, are preferred.

Tetracycline-responsive constructs comprise one or more tetracycline operator site ($TetO_2$) sequences, situated between the promoter and coding sequence. For example, two, three, four or even up to ten $TetO_2$ sequences may be used. Typically, if more than one $TetO_2$ site is used those sites will be arranged in the form of an array. However, other intervening nucleotide sequences may be situated between individual $TetO_2$ sites. For example one, two, three, four, five, up to ten or up to 15 nucleotides may intervene between any two $TetO_2$ sites.

The $TetO_2$ sequence is 5'-TCCCTATCAGTGATAGAGA-3' (Hillen and Berens, 1994, Annu. Rev. Microbiol. **48**, 345-369; Hillen *et al.*, 1983, J. Mol. Biol. **169**, 707-721) or a functional variant thereof. The $TetO_2$ sequence or a functional variant thereof is capable of being bound by a homodimer of tetR or a functional variant thereof.

A functional variant of the $TetO_2$ sequence is a sequence which is similar to that of the $TetO_2$ sequence and which remains capable of binding a homodimer of tetR or a functional variant thereof. The affinity of tetR for the $TetO_2$ sequence is $K_B = 2x10^{11}\ M^{-1}$ (as measured under physiological conditions), where K_B is the binding constant (Hillen and Berens, 1994, *supra*). The binding affinity of tetR for a functional variant of the $TetO_2$ sequence may be substantially the same as that of tetR for the $TetO_2$ sequence. Alternatively, tetR may have a binding affinity for a

functional variant of the $TetO_2$ sequence which is greater or less than that of tetR for the $TetO_2$ sequence. For example, the affinity of tetR for a functional variant of the $TetO_2$ sequence may be from $K_B = 2x10^9\ M^{-1}$ to $2x10^{13}\ M^{-1}$ or more preferably from $2x10^{11}\ M^{-1}$ to $2x10^{12}\ M^{-1}$.

A functional variant of $TetO_2$ typically comprises a sequence substantially similar to that of the $TetO_2$ sequence. Thus, a functional variant of $TetO_2$ will generally have at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98% or at least 99% sequence identity to the $TetO_2$ sequence, calculated over the full length of those sequences.

A functional variant of the $TetO_2$ sequence may be a modified version of that sequence obtained by, for example, nucleotide substitution or deletion. Up to 1, up to 2, up to 3, up to 4, up to 5, up to 6 or more nucleotide substitutions or deletions or combinations thereof may be made to the $TetO_2$ sequence to produce a functional variant of that sequence.

Constructs of the invention may be responsive to the antibiotic tetracycline or an analog thereof. Tetracycline binds to tetR homodimers, such that the tetR:tetracylcine complex dissociates from the $TetO_2$ sequence. The association constant of tetracycline to tetR is $3 \times 10^9\ M^{-1}$. Preferred analogs of tetracycline will have an association constant substantially similar to or greater than that of tetracycline for tetR. Suitable analogs of tetracycline include doxycycline. Doxycycline exhibits similar dose response and induction characteristics with constructs of the invention, but has a longer half-life than tetracycline (48 hours vs. 24 hours respectively).

The coding sequence used in both ecdysone- and tetracycline-responsive contructs of the invention can be any sequence which encodes a NOS or a functional variant thereof. The phrase "nitric oxide synthase" is intended to include all naturally occuring forms of iNOS, nNOS and eNOS as well as variants which retain NOS activity, for example variants produced by mutagenesis techniques. Preferably the coding sequence encodes a NOS of mammlian origin for example rodent (including rat and mouse) or human. Most preferably the coding sequence encode the human iNOS (GenBank accession number: X73029, Coding sequence 226-3687), human

nNOS (GenBank accession number: U17327, Coding sequence 686-4990) or human eNOS (GenBank accession number: M95296, Coding sequence 21-3632) or a functional variant of any one of those enzymes.

A functional variant of a NOS is any polypeptide which demonstrates NOS activity, for example a fragment of a NOS. A coding sequence which codes for a functional variant of a NOS may be, for example a fragment of a full length NOS coding sequence. A fragment may be of any length, so long as the polypeptide for which it codes has NOS activity.

A functional variant of a NOS typically comprises a sequence substantially similar to that of the naturally occurring form of the relevant NOS sequence. Thus, a functional variant of a NOS will generally have at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98% or at least 99% sequence identity to the relevant NOS sequence, calculated over the full length of those sequences.

Thus, the coding sequence may be modified by nucleotide substitutions or deletions. For example up to 1, 2 or 3 to 10, 25, 50, 75 or 100 substitutions or deletions or combinations thereof may be made to produce a functional variant of a NOS. A polynucleotide encoding a NOS may alternatively or additionally be modified by one or more insertions and/or deletions and/or by an extension at either or both ends. The modified polynucleotide generally encodes for a polypeptide which has NOS activity. Degenerate substitutions may be made and/or substitutions may be made which would result in a conservative amino acid substitution when the modified sequence is translated, for example as shown in the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other.

ALIPHATIC	Non-polar	G A P
		I L V
	Polar-uncharged	C S T M
		N Q
	Polar-charged	D E
		K R
AROMATIC		H F W Y

Sequence identity may be calculated as follows. The UWGCG Package provides the BESTFIT program which can be used to calculate identity (for example used on its default settings) (Devereux *et al* (1984) *Nucleic Acids Research* **12**, p387-395). The PILEUP and BLAST algorithms can be used to calculate identity or line up sequences (typically on their default settings), for example as described in Altschul S. F. (1993) J Mol Evol 36:290-300; Altschul, S, F *et al* (1990) J Mol Biol 215:403-10. Software for performing BLAST analyses is publicly available through the National Centre for Biotechnology Information (**http://www.ncbi.nlm.nih.gov/**).

(ii) Vectors

Both types of construct of the invention can be incorporated into a recombinant replicable vector. The vector may be used to replicate the construct in a compatible host cell. A vector may also provide for expression of the NOS coding sequence when the vector is harboured by an appropriate host cell. The vectors may be for example, plasmid, virus or phage vectors provided with an origin of replication and optionally a regulator of the ecdysone-responsive promoter or promoter used in a tetracycline-responsive construct.

The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene for selection in bacterial cells or a G418 or a zeocin resistance gene for selection in mammalian cells.

(iii) Cells

Vectors of the invention, which incorporate an ecdysone-inducible construct, may be introduced into a suitable host cell by any appropriate transformation or transfection technique.

Preferably, the host cell will permit the expression of the NOS coding sequence. Thus, the cells may be chosen to be compatible with the said vector and may be for example bacterial, yeast, insect or mammalian cells. For NOS gene expression to be induced in the presence of ecdysone or an analog thereof, a cell harbouring an ecdysone inducible construct must preferably also be capable of expressing a functional ecdysone receptor.

As described above, the wild type *Drosophila* functional ecdysone receptor is a heterodimer of the ecdysone receptor (EcR) and the product of the ultraspiracle gene (USP). Thus cells of the invention may be capable of expressing EcR and USP. However, replacement of EcR's natural heterodimeric partner USP with its mammlian homologue retinoid X receptor gives a heterodimer which can give more potent induction of an ecdysone responsive promoter. Thus cells of the invention may be capable of expressing EcR and RXR. It will be clear that cells of the invention may be capable of expressing functional variants of either subunit of the heterodimer. Functional variants of EcR and USP/RXR are polypeptides which can heterodimerise with their partner and can, when heterodimerised, allow ecdysone-responsive dimerisation to occur. In some cases functional variants may bind to non-wild type EcREs. Examples of functional variants and modified EcREs are described in No *et al. Proc. Natl. Acad. Sci. USA*, 93: 3346-3351.

Preferred cells for use in the invention are human cells. Particularly preferred cells are EcR293 cells (Invitrogen, San Diego, CA; Catalogue No: R650-07; EcR293 is a derivative of the human fetal kidney cell line HEK293 (ECACC accession number 85/20602)). EcR293 cells are particularly suitable as they stably transformed with the vector pVgRXR. That vector is capable of expressing a functional variant of EcR, VgEcR, and RXR in mammalian cells and thus allows the expression of a functional ecdysone receptor. Other suitable cell lines include EcR-CHO and EcR-3T3 (Invitrogen, San Diego, CA; Cat. Nos: R660-07 and R680-07 respectively).

Those two cell lines are stably transformed with the same vector, pVgRXR, as the EcR293 cell line.

Vectors of the invention, which incorporate an tetracycline-inducible construct, may be introduced into a suitable host cell by any appropriate transformation or transfection technique.

Preferably, the host cell will permit the expression of the NOS coding sequence in the presence of tetracycline or an analog thereof. Thus, the cells may be chosen to be compatible with the said vector and may be for example bacterial, yeast, insect or mammalian cells. For NOS gene expression to be regulated such that expression does not occur in the absence of tetracycline, a cell harbouring a tetracycline-inducible construct must preferably also be capable of expressing the tetracycline repressor protein (tetR) or a functional variant thereof.

A functional variant of tetR is a polypeptide which is similar to tetR and which remains capable of binding, as a homodimer, the $TetO_2$ site or a functional variant thereof and tetracycline or an analog thereof. Typically, the binding affinity of a functional variant sequence of tetR for the $TetO_2$ site or a functional variant thereof or tetracycline or an analog thereof may be substantially the same as the binding affinity of the tetR polypeptide for the $TetO_2$ site or a functional variant thereof or tetracycline or an analog thereof. Alternatively, a functional variant sequence may have a binding affinity which may be greater or less than that of the tetR polypetide.

The *TetR* gene encodes a repressor protein of 207 amino acids with a calculated molecular weight of 23 kDa (Hillen and Berens, 1994, *supra*). A functional variant of tetR typically comprises an amino acid sequence substantially similar to that of the tetR sequence. Thus, a functional variant of a tetR will generally have at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98% or at least 99% sequence identity to tetR, calculated over the full length of those sequences. The calculation of sequence identities is described above.

A functional variant of the tetR sequence may be a modified version of that sequence obtained by, for example, amino acid substitution or deletion. Up to 1, up to 10, up to 20, up to 50, up to 75, up to 100 or more amino acid substitutions or

deletions or combinations thereof may be made to the tetR sequence to produce a functional variant of that sequence. Substitutions are preferably made which result in a conservative amino acid substitution, for example as shown in the Table above.

Preferred cells for use in the invention are human cells. Particularly preferred cells are T-REx cells (Invitrogen, San Diego, CA; Catalogue Nos: R710-07, R712-07, R714-07 and R716-07). T-Rex cells are particularly suitable as they stably transformed with the plasmid pcDNA6/TR which generates high level expression of the tetR polypeptide. However, any cell line can be used which expresses tetR or a functional variant thereof.

Assays

The principle behind the assays of the invention is to screen for compounds which modulate NOS activity. The assays can also be used to screen for compounds which specifically modulate only one of the NOS isoforms. Thus, the invention provides a method for identifying a modulator of NOS activity comprising:

(i) providing a polynucleotide construct which comprises:

(a) a promoter operably linked to a coding sequence, wherein the promoter is responsive to ecdysone or an analog thereof and the coding sequence encodes a nitric oxide synthase (NOS) or a functional variant thereof; or

(b) a promoter operably linked to one or more tetracycline operator site sequences and a coding sequence in that order, wherein the coding sequence encodes a nitric oxide synthase (NOS) or a functional variant thereof;

(ii) contacting a test substance with the construct under conditions that would permit the expression and activity of the NOS encoded by the coding sequence in the absence of the test substance; and

(iii) determining thereby whether the said substance modulates NOS activity.

In step (i) of the above method a construct can be incorporated into a vector and the resulting vector can be contacted with the test substance as described in the

above method. Preferably, a construct of the invention or a vector incorporating a construct of the invention may be harboured by a cell and that cell may be contacted with the test substance.

Any suitable assay format may be used for identifying a modulator of NOS activity. The assay is, however, typically carried out in a single medium. Most preferably the assay is carried out in a single well of a plastics microtitre plate, so that high through-put screening for modulators of NOS activity may be carried out.

In practice, cells may be grown in the presence of the test substance and ecdysone or an analog thereof or tetracycline or an analog thereof depending on the construct used. Enzymatic action of NOS may be commenced by the addition of a NOS substrate, for example L-arginine. The activity of the NOS is then determined.

Various control experiments may be appropriate. The progress of the assay can be followed in the absence of the test substance. Known NOS modulators, eg. the NOS inhibitor L-N^6-(1-iminoethyl)lysine (L-NMMA) or the NOS activator tetrahydrobiopterin (BH_4) may be used as positive controls, in order to show that the expressed enzymes are indeed capable of modulation. Also, a test substance may be tested with any other known enzyme to exclude the possibility that the test substance is a general modulator of enzyme activity.

The reaction mixture will typically contain a suitable buffer, suitable cofactors and suitable cations as cofactors. A suitable buffer includes any suitable biological buffer that can provide buffering capability at a pH conducive to the reaction requirements of NOS enzymes. All three enzymes require NADPH, FMN, FAD, BH_4 and calmodulin for activity. eNOS and nNOS also require calcium. The assay may be carried out at any temperature at which a NOS, in the absence of any inhibitor, is active. Typically, however, the assay will be carried out in the range of from 25°C to 37°C.

Measures of the enzymatic activity of a NOS will be generally known to those skilled in the art, including equilibrium constants, reaction velocities of the appearance of reaction products or the consumption of reaction substrates, reaction kinetics, thermodynamics of reaction, spectrophotometric analysis of reaction products, detection of labelled reaction components, etc. See generally, Segel,

Biochemical Calculations, 2nd Edition, John Wiley and Sons, New York (1976); Suelter, A Practical Guide to Enzymology, John Wiley and Sons, New York (1985).

NOS activity can be measured in a number of ways (*Methods in Enzymology* **268**, L. Packer (Ed.) Academic Press, 1996). For example, NOS activity can be measured directly by conversion of radiolabelled arginine to citrulline or indirectly by assaying for NO production. Assays for NO include chemical assays and bioassays. A typical assay for NO is described in Green *et al.*, 1982, Analysis of nitrate, nitrite and [^{15}N] nitrate in biological fluids, *Anal. Chem.* **126**, 131-138. An alternative chemical assay for NO utilises the change in absorbance at 405nm and 420nm when NO oxidises oxyhaemoglobin to methemoglobin. That assay is described in Charles *et al.*, 1996, *Methods in Enzymology* **268**: 449-460). Both the above examples of assays for NO are suitable for use in a microtitre plate format.

A typical assay is as follows:

- cells harbouring a vector of the invention are inoculated in a suitable growth medium into the wells of a plastics micro-titre plate and grown to semi-confluence or confluence;
- muristone A is added to a final concentration of 1-10μM or tetracycline is added to a final concentration of about 0.025μg/ml along with the substance or pool of substances to be tested and the cells are incubated overnight;
- 100μl of the culture supernatant is mixed with 100μl of Griess reagent (1:1 mixture of 1% sulfanilamide in 5% H_3PO_4 and 0.1% napthylethylenediamine dihydrochloride in water) for 10 min at room temperature and absorbance at 543nm is measured; and
- serial dilutions of sodium nitrite can be used to generated standards and inhibition of NOS determined by the comparison of sodium nitrate formed in the absence of the test substance and in the presence of the test substance.

Modulators

Suitable test substances which can be tested in the above assays include combinatorial libraries, defined chemical identities, peptide and peptide mimetics, oligonucleotides and natural product libraries, such as display libraries (eg. phage display libraries) and antibody products (for example, monoclonal and polyclonal

antibodies, single chain antibodies, chimaeric antibodies and CDR-grafted antibodies) which are specific for NOS enzymes in general or specific for one of the NOS isoforms or mimics thereof. Other suitable test substances include analogs of arginine, aminoguanidine or analogues thereof, guanidine compounds, isothiourea, derivatives of L-N^6-(1-iminoethyl)lysine (L-NMMA), 2-nitroaryl and 2-cyanoaryl compounds, δ-(S-methylisothioureido)-L-norvaline and BH_4, ie. analogs of known inhibitors and activators of NOS.

Test substances may be used in an initial screen of, for example, ten substances per reaction, and the substance of these batches which show inhibition or activation tested individually. Test substances may be used at a concentration of from 1μM to 1000μM, preferably from 1μM to 100μM, more preferably from 1μM to 10μM.

A substance which inhibits or activates the activity of NOS may do so by binding the enzyme. Such enzyme inhibition or activation may be reversible or irreversible. An irreversible inhibitor or activator dissociates very slowly from its target enzyme because it becomes very tightly bound to the enzyme, either covalently or non-covalently. Reversible inhibition or activation, in contrast with irreversible inhibition or activation, is characterised by a rapid dissociation of the enzyme-inhibitor/activator complex.

The test substance may be a competitive inhibitor. In competitive inhibition, the enzyme can bind substrate (forming an enzyme-substrate complex) or inhibitor (enzyme-inhibitor complex) but not both. Many competitive inhibitors resemble the substrate and bind the active site of the enzyme. The substrate is therefore prevented from binding to the same active site. A competitive inhibitor diminishes the rate of catalysis by reducing the proportion of enzyme molecules bound to a substrate.

The inhibitor may also be a non-competitive inhibitor. In non-competitive inhibition, which is also reversible, the inhibitor and substrate can bind simultaneously to an enzyme molecule. This means that their binding sites do not overlap. A non-competitive inhibitor acts by decreasing the turnover number of an enzyme rather than by diminishing the proportion of enzyme molecules that are bound to substrate.

The inhibitor can also be a mixed inhibitor. Mixed inhibition occurs when an inhibitor both effects the binding of substrate and alters the turnover number of the enzyme.

A substance which inhibits the activity of NOS may also do so by binding to the substrate. The substance may itself catalyse a reaction of the substrate, so that the substrate is not available to the enzyme. Alternatively the inhibitor may simply prevent the substrate binding to the enzyme.

A substance which is an activator may increase the affinity of the substrate for the enzyme or *vice versa*. If this is the case the proportion of enzyme molecules bound to substrate molecules is increased and the rate of catalysis will thus increase. An activator may increase the affinity of a substrate for an enzyme by binding to the enzyme or substrate or both.

A modulator of NOS activity is one which produces a measurable reduction or increase in NOS activity in the assays described above.

Preferred inhibitors are those which inhibit NOS activity by at least 10%, at least 20%, at least 30%, at least 40% at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 99% at a concentration of the inhibitor of 1μg ml^{-1}, 10μg ml^{-1}, 100μg ml^{-1}, 500μg ml^{-1}, 1mg ml^{-1}, 10mg ml^{-1}, 100mg ml^{-1}.

Preferred activators are those which activate NOS activity by at least 10%, at least 25%, at least 50%, at least 100%, at least, 200%, at least 500% or at least 1000% at a concentration of the activator 1μg ml^{-1}, 10μg ml^{-1}, 100μg ml^{-1}, 500μg ml^{-1}, 1mg ml^{-1}, 10mg ml^{-1}, 100mg ml^{-1}.

The percentage inhibition or activation represents the percentage decrease or increase in activity of NOS in a comparison of assays in the presence and absence of the test substance. Any combination of the above mentioned degrees of percentage inhibition or activation and concentration of inhibitor or activator may be used to define an inhibitor or activator of the invention, with greater inhibition or activation at lower concentrations being preferred.

Candidate substances which show activity in assays such as those described above can be tested in *in vivo* systems. For example, the modulation of eNOS and iNOS *in situ* in rat aortic rings can assessed by measuring the change in ring tension

caused by candidate substances. For studies of basal tone, rings of thoracic aorta with intact endothelium are typically prepared as described in Rees *et al.* (1989, *Br. J. Pharmacol.* 96; 418-424) and cumulative concentration curves obtained for the test substances in the presence of a threshold concentration of phenylephrine (ED_{10}=10nM). For studies of induced smooth muscle tone, endothelium-denuded rings are typically exposed to LPS (0.1 μg/ml from *S. thphosa*) in the presence of phenylephrine at approximately ED_{90} for 6 h as described in Rees *et al.* (1990). During this time a progressive loss of tone usually occurs because of iNOS induction. Generally, cumulative concentration curves are then obtained for the candidate substances.

The inhibition or activation of eNOS and iNOS *in vivo* can also be assessed by the effects of inhibitors on blood pressure in mice. For example, one suitable method is as follows. Mice are anaesthetised briefly with isoflourane (2%). Cannula lines are implanted in a femoral vein, tunnelled subcutaneously to exit at the top of the back and connected to a swivel tether system for continuous monitoring of blood pressure and for candidate substance administration respectively. Following recovery from surgery, animals with mean blood pressures in the normal range (90-110 mm Hg) are used to obtain cumulative concentration curves for candidate substances on blood pressure. It will be appreciated that other suitable assays may be used to determine whether a candidate substance shows modulation of NOS activity *in vivo* and that the above mentioned assays are by way of example.

Ultimately, candidate modulators may be tested in an animal model of target disease state.

Therapeutic Uses

Modulators of NOS activity can be used in the treatment of conditions in which the abnormal metabolism of nitric oxide is implicated. Thus, the invention also provides use of a modulator of NOS activity for the manufacture of a medicament for use in the treatment of a clinical condition associated with abnormal NO production.

Excessive production of NO as a result of, for example, the effects of

bacterial endotoxins, can lead to potentially fatal vasodilation and hypotension. Modulators identified by a method of the invention which are inhibitors of NOS activity may be used to prevent, alleviate or treat this potentially fatal response.

Modulators of the invention which are inhibitors may also be used to treat other conditions in which over-production of NOS is implicated, for example ischaemia-reperfusion injury of the brain or heart, cancer, multiple sclerosis, lethal hypotension in severe inflammatory conditions such as septic shock or multi-organ failure, or local and systemic inflammatory disorders including arthritis, skin disorders, inflammatory cardiac disease or inflammatory bowel disease. Over-production of NOS is also implicated in various neurological disorders including strokes, dementia, Parkinson's disease and Alzheimer's disease. The condition of a patient suffering from one of the above mentioned conditions can therefore be improved by administration of a modulator of the invention which is an inhibitor of NOS activity. A therapeutically effective amount of such a modulator may be given to a human patient in need thereof.

Deficient production is implicated in a number of clinical conditions including, for example, hyperlipidaemia, renal failure, hypertension, restenosis after angioplasty, atherosclerosis, complications of heart failure, schizophrenia and cancer. Modulators of the invention which are activators of NOS activity may be used to prevent, alleviate or treat any one of those conditions. The condition of a patient suffering from one of the above mentioned conditions can therefore be improved by administration of such a modulator. A therapeutically effective amount of such a modulator may be given to a human patient in need thereof.

Modulators of NOS activity may be administered in a variety of dosage forms. Thus, they can be administered orally, for example as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules. The inhibitors may also be administered parenterally, either subcutaneously, intravenously, intramuscularly, intrasternally, transdermally or by infusion techniques. The modulators may also be administered as suppositories. A physician will be able to determine the required route of administration for each particular patient.

The formulation of a modulator for use in prophylaxis or treatment will depend upon factors such as the nature of the exact modulator, whether a pharmaceutical or veterinary use is intended, etc. An modulator may be formulated for simultaneous, separate or sequential use.

A modulator of NOS activity is typically formulated for administration in the present invention with a pharmaceutically acceptable carrier or diluent. The pharmaceutical carrier or diluent may be, for example, an isotonic solution. For example, solid oral forms may contain, together with the active compound, diluents, e.g. lactose, dextrose, saccharose, cellulose, corn starch or potato starch; lubricants, e.g. silica, talc, stearic acid, magnesium or calcium stearate, and/or polyethylene glycols; binding agents; e.g. starches, arabic gums, gelatin, methylcellulose, carboxymethylcellulose or polyvinyl pyrrolidone; disaggregating agents, e.g. starch, alginic acid, alginates or sodium starch glycolate; effervescing mixtures; dyestuffs; sweeteners; wetting agents, such as lecithin, polysorbates, laurylsulphates; and, in general, non-toxic and pharmacologically inactive substances used in pharmaceutical formulations. Such pharmaceutical preparations may be manufactured in known manner, for example, by means of mixing, granulating, tabletting, sugar-coating, or film coating processes.

Liquid dispersions for oral administration may be syrups, emulsions and suspensions. The syrups may contain as carriers, for example, saccharose or saccharose with glycerine and/or mannitol and/or sorbitol.

Suspensions and emulsions may contain as carrier, for example a natural gum, agar, sodium alginate, pectin, methylcellulose, carboxymethylcellulose, or polyvinyl alcohol. The suspensions or solutions for intramuscular injections may contain, together with the active compound, a pharmaceutically acceptable carrier, e.g. sterile water, olive oil, ethyl oleate, glycols, e.g. propylene glycol, and if desired, a suitable amount of lidocaine hydrochloride.

Solutions for intravenous or infusions may contain as carrier, for example, sterile water or preferably they may be in the form of sterile, aqueous, isotonic saline solutions.

A therapeutically effective amount of a modulator is administered to a

patient. The dose of modulator may be determined according to various parameters, especially according to the substance used; the age, weight and condition of the patient to be treated; the route of administration; and the required regimen. Again, a physician will be able to determine the required route of administration and dosage for any particular patient. A typical daily dose is from about 0.1 to 50 mg per kg, preferably from about 0.1mg/kg to 10mg/kg of body weight, according to the activity of the specific inhibitor, the age, weight and conditions of the subject to be treated, the type and severity of the degeneration and the frequency and route of administration. Preferably, daily dosage levels are from 5 mg to 2 g.

The following Examples illustrates the invention:

Examples

Materials and methods

Unless indicated otherwise, the methods used are standard biochemical techniques. Examples of suitable general methodology textbooks include Sambrook *et al.*, Molecular Cloning, a Laboratory Manual (1989) and Ausubel *et al.*, Current Protocols in Molecular Biology (1995), John Wiley & Sons, Inc.

Subculture Procedure

All of the operations were carried out under strict aseptic conditions in a laminar flow hood. The medium was removed from near-confluent flasks and gently washed twice with serum-free DMEM (Dulbecco's Modified Eagle's Medium). A trypsin/versene mix was added to the cells and they were incubated for 5-10 min. until the cells had detached. Once the cells had detached they were resuspended in pre-warmed (37°C) serum-free DMEM and then pelleted by centrifugation at 1000rpm for 5min. It was essential to wash the cells to remove the residue of the tyrpsin/versene (serum-free DMEM was used for washing). The supernatant was removed and the cells gently resuspended in 15ml of 90% DMEM with glutamine, 10% fetal bovine serum (FBS, cell culture grade) that had been pre-warmed to 37°C. The cells were then transferred to three or four T-25 flasks (or equivalent) and placed

in a humidified, 37°C, 5% CO_2 incubator. It typically took 3-4 days for cell cultures to reach 80-90% confluency. Media were changed 2 times a week and cells subcultured at a ratio of 1:3 to 1:4 when they reached 80-90% confluency. Cells were subcultured when approaching confluency to avoid the accumulation of floating and dead cells. Cells were frozen for storage in 95% FBS and 5% DMSO.

Induction using Muristerone A

Cells could be maintained in non selection medium for 2-3 weeks without losing inducibility following muristerone A treatment. Non-selection medium was used by choice, although selection medium worked equally well. Cells were seeded at 1-2 x 10^5/ml concentration for 12 well or 96 well plates. Semi-confluent or confluent plates or flasks were used for induction. Figure 1(a) shows overnight induction in 12 well plates (3-30 hours) following the addition of different doses of muristerone A. Nitrate concentrations are measured using the Griess Reaction (see below).

Western blot analysis

Cells were pelleted at 200g, followed by two washes in ice-cold PBS, pH 7.2, then resuspended in the extraction buffer (50mM NaF, 20mM Hepes (pH 7.8), 450mM NaCI, 25% (vol/vol) glycerol, 0.2mM EDTA, 0.5mM dithiothreitol, 0.5mM phenylmethylsulfonyl fluoride, leupeptin (0.5µg/ml), protease inhibitor (0.5µg/ml), trypsin inhibitor (1.0µg/ml), aprotinin (0.5µg/ml), bestatin (40µg/ml)) and left on ice for 10 min. Following centrifugation at 10,000g for 10 min at 4°C, the supernatant was collected and the cell extract assayed for protein using the BCA kit (Pierce). One fifth of a volume of five times sample buffer (0.25M Tris-HCL (pH6.8), 0.4M DTT, 5% SDS, 0.5% bromophenol blue, 50% glycerol) was added to each sample and boiled for 5 min prior to storage at -70°C. Electrophoresis was carried out on 6% SDS polyacrylamide gels with 25µg samples. Proteins were transferred to polyvinylidine difluride (PVDF) membranes (Amersham) and immunoblotting carried out with the appropriate antibody using ECL (Amersham). Where necessary, blots were stripped in 62.5mM Tris-HCL 100mM β-mercaptoethanol/ 2% SDS, (pH

6.7) and reprobed with different antibodies.

Northern Blotting

Poly(A) + mRNA was isolated using a micro-fastrack mRNA purification kit (Invitrogen), separated by electrophoresis and transferred onto Hybond N membrane (Amersham). Phosphoimaging (BAS1000, Fujix) was used to quantify the signals using the MacBas image analysis software.

NOS activity assay

Griess Reaction (Green *et al.*, 1982, Analysis of nitrate, nitrite and [^{15}N] nitrate in biological fluids, *Anal. Chem.* **126**, 131-138): NOS activity was determined for both intact cells and their lysates. For intact cells, 100µl of the culture medium was mixed with 100µl of Griess reagent (1:1 mixture of 1% sulfanilamide in 5% H_3P0_4 and 0.1% naphthylethyethylenediamine dihydrochloride in water) for 10 min at room temperature and the absorbance at 543 nm was recorded. A serial dilution of sodium nitrite was used as a standard.

For enzyme assays on cell lysates, 100µg of lysate was mixed with 100µl reaction reagent from the NOS detect system (Stratagene NOS detect kit, Cat. No. 204500). The kit measures the conversion of [^{14}C]arginine to [^{14}C] citrulline, and is specific for the NOS pathway.

Example 1 - Generation of NOS transfected cell lines under the control of an ecdysone-responsive or a tetracycline-responsive promoter

Three plasmids were generated, each of which expressed one of the human NOS isoforms under the control of an ecdysone-responsive promoter. An additional plasmid was constructed placing the iNOS cDNA under the control of a tetracycline-regulated promoter.

(I) pIND-hiNOS-f (Figure 1a)

4164bp of the human iNOS cDNA (GenBank accession number: X73029, Coding sequence 226-3687) was cut from its original vector (Bluescript KS) using

the restriction endonucleases, *Kpn*I and *Spe*I and cloned into pIND (Invitrogen, San Diego, CA; Catalogue No: V705-20) which had been cut with *Kpn*I and *Xba*I. The resulting plasmid was sequenced to confirm that the cDNA had inserted in the correct orientation.

(II) pIND-hnNOS-f (Figure 1b)

5kb of the human nNOS cDNA (GenBank accession number: U17327, Coding sequence 686-4990) was cut from its original vector (Bluescript KS) using the restriction endonucleases, *Xba*I and *Kpn*I and cloned into pIND (Invitrogen, San Diego, CA; Catalogue No: V705-20) which had been cut with *Nhe*I and *Kpn*I. The resulting plasmid was sequenced to confirm that the cDNA had inserted in the correct orientation.

(III) pIND-heNOS-f (Figure 1c)

The wild type human eNOS (GenBank accession number: M95296, Coding sequence 21-3632) was cut from its original vector (Bluescript KS) using the restriction endonucleases, *Hind*III and *Not*I and cloned into pIND (Invitrogen, San Diego, CA; Catalogue No: V705-20) which had been cut with *Hind*III and *Not*I. The resulting plasmid was sequenced to confirm that the cDNA had inserted in the correct orientation.

(IV) pTet-hiNOS-f (Figure 1d)

The human iNOS cDNA (GenBank accession number: X73029, Coding sequence 226-3687) was cut from its original vector (Bluescript KS) using the restriction endonucleases, *Kpn*I and *Spe*I and cloned into pcDNA4/TO (Invitrogen, San Diego, CA) which had been cut with *Kpn*I and *Xba*I. The resulting plasmid was sequenced to confirm that the cDNA had inserted in the correct orientation.

The pIND plasmid contains 5 modified EcREs called E/GREs which bind a modified functional ecdysone receptor. That modified functional ecdysone receptor can be expressed by another plasmid, pVgRXR (Invitrogen, San Diego, CA;

Catalogue Number: V730-20). pVgRXR constitutively expresses a heterodimeric receptor comprising a modified ecdysone receptor (VgEcR) and RXR. Thus, a cell transformed with pVgRXR and one of the three plasmids described above (I, II or III) will express NOS in the presence of ecdysone or an analog thereof. In the presence of ecdysone the functional ecdysone receptor binds to the E/GREs and transcription of the NOS cDNA is initiated.

The plasmid pIND-hiNOS-f was used to transfect a human fetal kidney cell line, EcR293 (Invitrogen, San Diego, CA; Catalogue No: R650-07), which is stably transformed with pVgRXR. Transfections were carried out using Superfect reagent (Qiagen) and transfectants were isolated following double selection on G418 (400μg/ml) and zeocin (250μg/ml) for 14 days.

Thus, cells were isolated which constitutively expressed the subunits of a functional ecdysone receptor, RXR/VgEcR and the human iNOS cDNA under the control of an ecdysone-inducible promoter.

The T-Rex system (Invitrogen, San Diego, CA; Catalogue No: K1020-01) is a tetracycline-regulated mammalian expression system that uses regulatory elements from the *E. coli* Tn*10*-encoded tetracycline resistance operon. The pcDNA4/TO plasmid allows expression of a gene of interest under te control of the strong human cytomegalovirus immediate-early (CMV) promoter and two tetracycline operator 2 ($TetO_2$) sites. The pcDNA6/TR plasmid expresses high levels of the *Tet*R gene under the control of the human CMV promoter. Thus, a cell transformed with pcDNA6/TR and the plasmid described above (IV) will express NOS in the presence of tetracycline or an analog thereof. When present, tetracycline binds to tetR which undergoes a conformational change such that it dissociates from the $TetO_2$ sites. Expression of the iNOS gene is then induced, driven by the CMV promoter.

The plasmid pTet-hiNOS-f was used to transfect the cell line T-REx-293 ((Invitrogen, San Diego, CA; Catalogue No: R710-07). The T-REx-293 cell line is a human embryonic kidney 293 cell line which has been transfected with the pcDNA6/TR plasmid and thus generates high level expression of the tetracycline repressor protein (TetR). Transfections were carried out using conditions as

described above for the muristerone A inducible constructs. Transfectants were isolated following selection on zeocin (200µg/ml) for the human iNOS cDNA expressing plasmid and blasticidin (5µg/ml) for the tetR expressing plasmid.

Thus, cells were isolated which constitutively expressed the tetracycline repressor protein (tetR) and the human iNOS cDNA in the presence of tetracycline.

Example 2 - Isolation and characterization of ecdysone-responsive and tetracycline-responsive human cell lines

(A) Isolation of a panel of ecdysone-responsive human cell lines and determination of NOS activity

A panel of 20 G418/zeocin-resistant clones were examined for their ability to generate NO. A total of 5 were identified that could be induced to produce NO at varing levels after treatment with 100µM muristerone A for 24 hours. NOS activity was determined in both intact cells and in cell lysates. For intact cells, the Griess reaction was used to determine the concentration of NO in 100µl of culture medium. For enzyme assays, 100µg of cell lysate was mixed with 100µl reaction reagent from NOS detect system (Stratagene NOS detect kit, Cat. No.204500). The kit measures the conversion of [^{14}C] arginine to [^{14}C] citrulline, and is specific for NOS. For a typical experiment, transfectants were plated out on 12 well Falcon tissue culture plates at a cell density of 1 x 10^5/ml and nitrite was measured by the Griess reaction. Muristerone A (Invitrogen) was added at a final concentration of 100µM to specific wells, and after induction for 24hr, 100µl of culture supernatant was used to measure nitrite concentration using the Griess reagent. The results are reported as the average of assays run on triplicate wells. Well-to-well variation was less than 10%.

(B) Time and dose response of EcR293 clone 11 cells generating NO

One of the transfectants, clone 11, was selected for further study. Cells were grown with varying concentrations of muristerone A, and at different time intervals, supernatants were taken and the Griess reaction was used to measure the nitrite concentration. The results are reported as the average of assays run in triplicate. Well-to-well variation was less than 10%. See Figure 2a.

(i) Muristerone A-dependent expression of the human iNOS gene. Northern blot analysis was carried out with 2μg of polyA+ RNA isolated from cells which had been treated with muristerone A for 24 hr. A human iNOS cDNA probe was used to detect the presence of a 4kb band in mRNA extracted from cells treated with either 1μM or 10μM muristerone A. Human β-actin mRNA was used as a loading control. See Figure 2b.

(ii) Western blot of iNOS protein expression was carried out on untreated control cells or cells treated with 10μM muristerone A. The cells were harvested and 20μg of whole cell extracts loaded on to a 6% polyacrylamide gel. Following electrophoresis, the proteins were transferred to a filter and probed with a polyclonal antibody raised against the 7 C-terminal residues of human iNOS: Cyc-Arg-Nle-Orn-(Ser-Leu-Glu-Met-Ser-Ala-Leu). Filters were stripped and an antibody against human alpha-tubulin. (Insight Biotchnology) was used as a control. See Figure 2b.

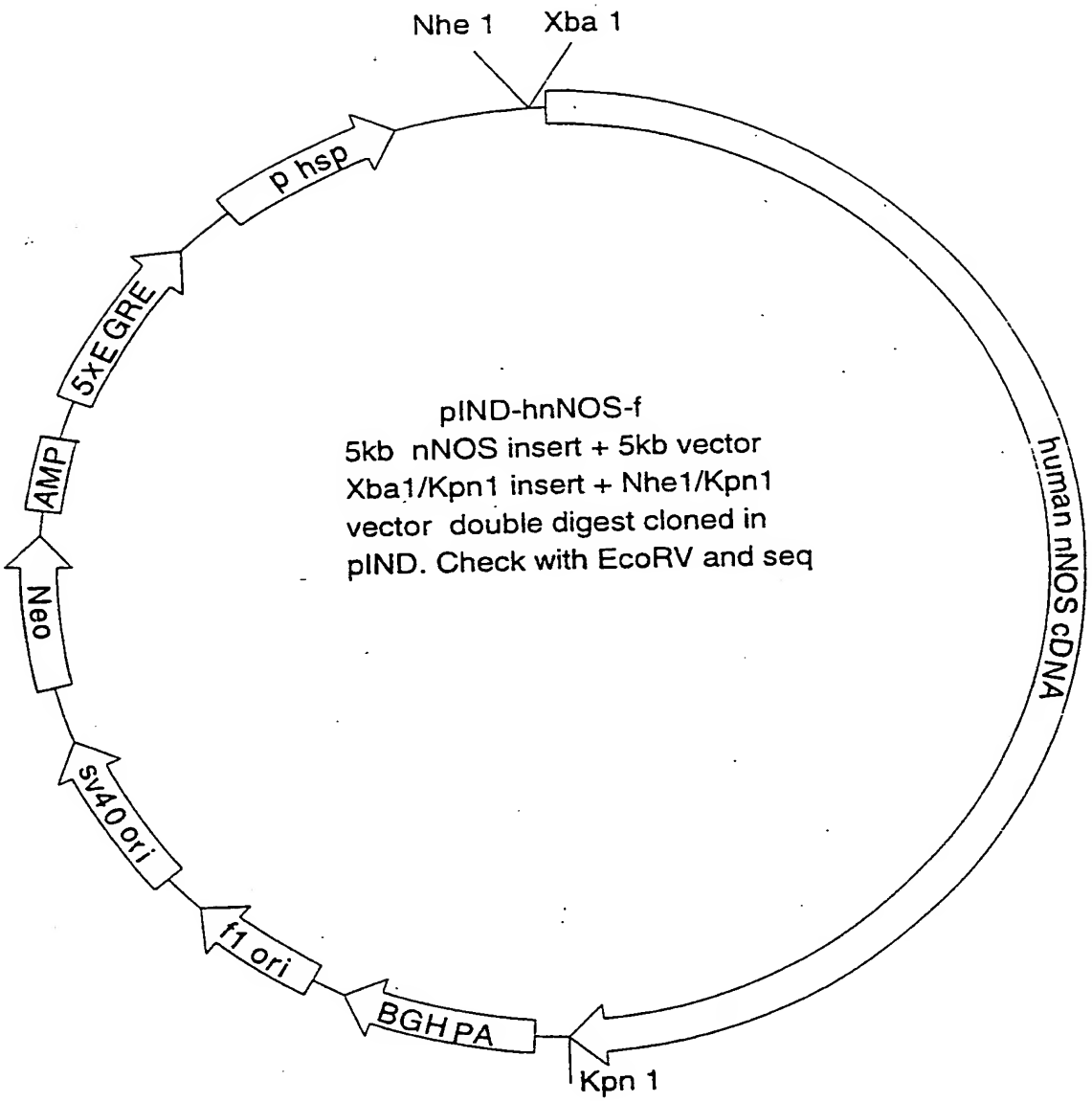
(C) Isolation of a panel of tetracycline-responsive human cell lines and determination of NOS activity

A panel of 4 tetracycline-regulated iNOS expressing cell lines were isolated, designated clone 1, clone 2, clone 5 and clone 22.

In the absence of the inducer tetracycline the iNOS transfectants were unable to express mRNA as the two Tet operator sites ($Tet0_2$) are occupied by the repressor protein effectively blocking transcription. When tetracycline is added to the culture medium, it binds to the TetR protein and changes its conformation. The altered conformation of the repressor is unable to bind the operator sites, and consequently iNOS can be expressed. Nitrite concentrations were determined using the Griess reaction (see figure 3).

Example 3 - Screening for inhibitors of human iNOS using the EcR293 clone 11 cell line

Cells were seeded at 1-2x10^5/ml concentration for 12 well or 96 well plates. Semi-confluent or confluent plates or flasks were used for induction with muristerone A (1-10μM). To test iNOS inhibitors, the test compounds were dissolved in an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Xba 1
p hsp
5xEGRE
AMP
Neo
sv40 ori
f1 ori
BGH PA
Kpn 1
human nNOS cDNA
pIND-hnNOS-f
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vector double digest cloned in
pIND. Check with EcoRV and seq

Figure 1b

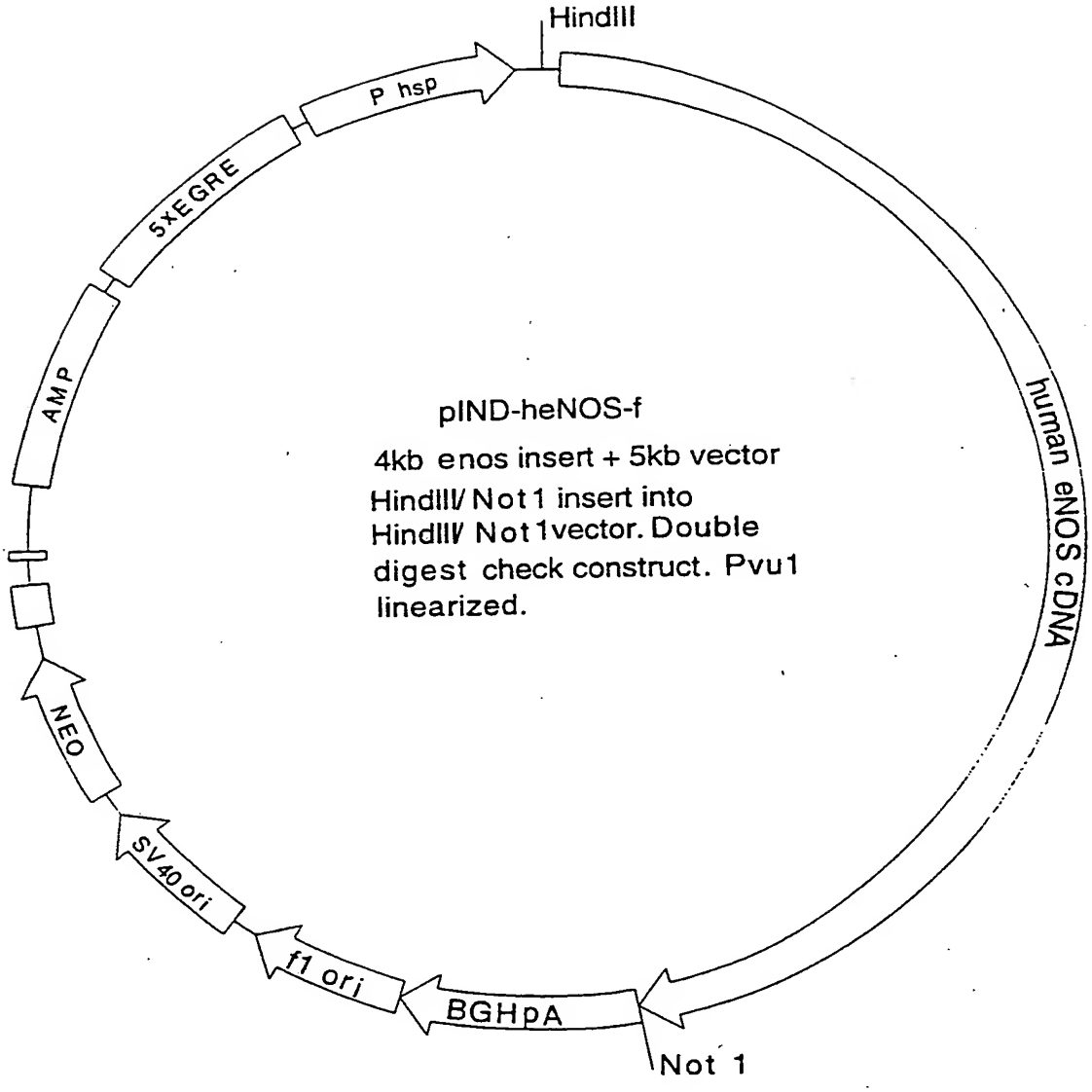

HindIII
P hsp
5XEGRE
AMP
human eNOS cDNA
pIND-heNOS-f
4kb enos insert + 5kb vector
HindIII/ Not 1 insert into
HindIII/ Not 1vector. Double
digest check construct. Pvu1
linearized.
NEO
SV40 ori
f1 ori
BGHpA
Not 1

Figure 1c

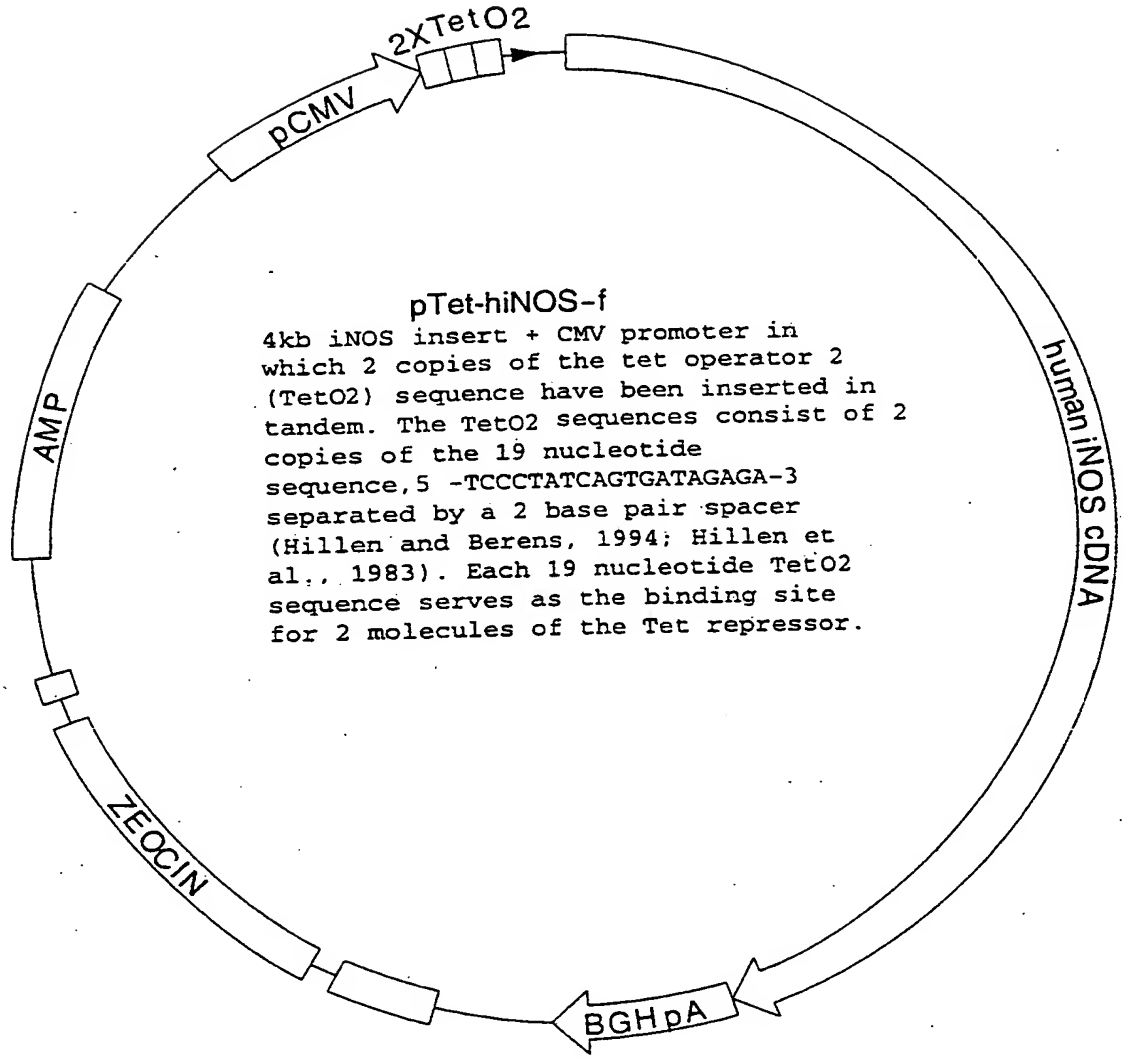
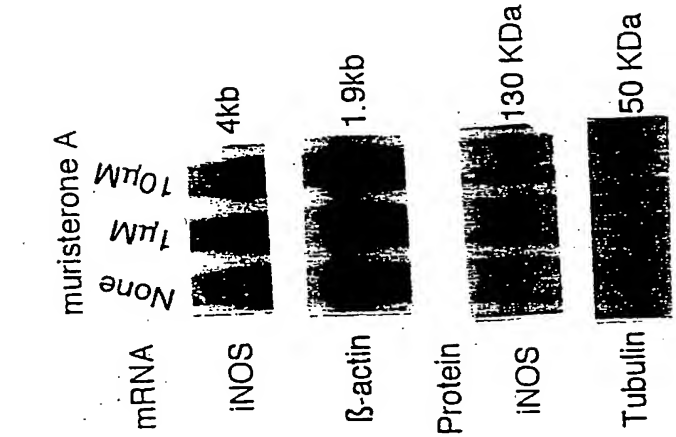

2XTetO2
pCMV
AMP
human iNOS cDNA
ZEOCIN
BGHpA
pTet-hiNOS-f
4kb iNOS insert + CMV promoter in which 2 copies of the tet operator 2 (TetO2) sequence have been inserted in tandem. The TetO2 sequences consist of 2 copies of the 19 nucleotide sequence, 5 -TCCCTATCAGTGATAGAGA-3 separated by a 2 base pair spacer (Hillen and Berens, 1994; Hillen et al., 1983). Each 19 nucleotide TetO2 sequence serves as the binding site for 2 molecules of the Tet repressor.

Figure 1d


muristerone A
None
1μM
10μM
mRNA
iNOS
4kb
ß-actin
1.9kb
Protein
iNOS
130 KDa
Tubulin
50 KDa

b.

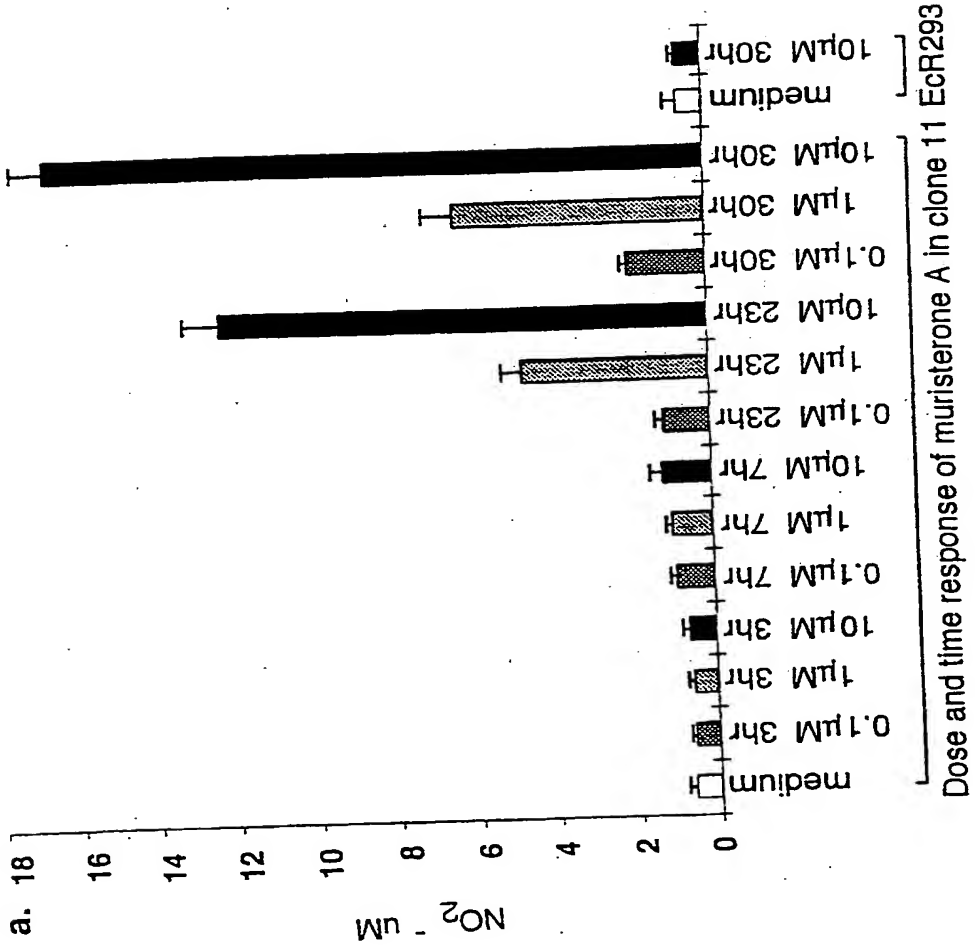

a.
NO2 - uM
0
2
4
6
8
10
12
14
16
18
medium
0.1μM 3hr
1μM 3hr
10μM 3hr
0.1μM 7hr
1μM 7hr
10μM 7hr
0.1μM 23hr
1μM 23hr
10μM 23hr
0.1μM 30hr
1μM 30hr
10μM 30hr
medium
10μM 30hr
Dose and time response of muristerone A in clone 11 EcR293

Figure 2

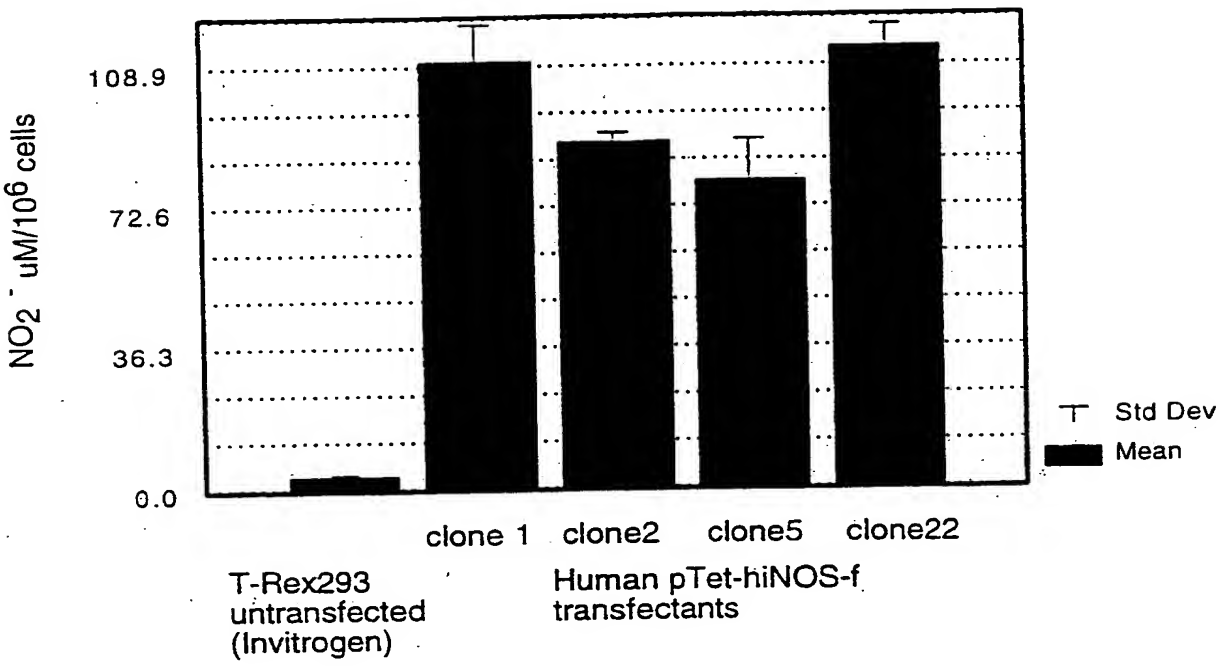

108.9
72.6
36.3
0.0
NO_2^- uM/10^6 cells
clone 1
clone2
clone5
clone22
T-Rex293 untransfected (Invitrogen)
Human pTet-hiNOS-f transfectants
Std Dev
Mean
induced with tetracycline at 1ug/ml 24 hours

Figure 3

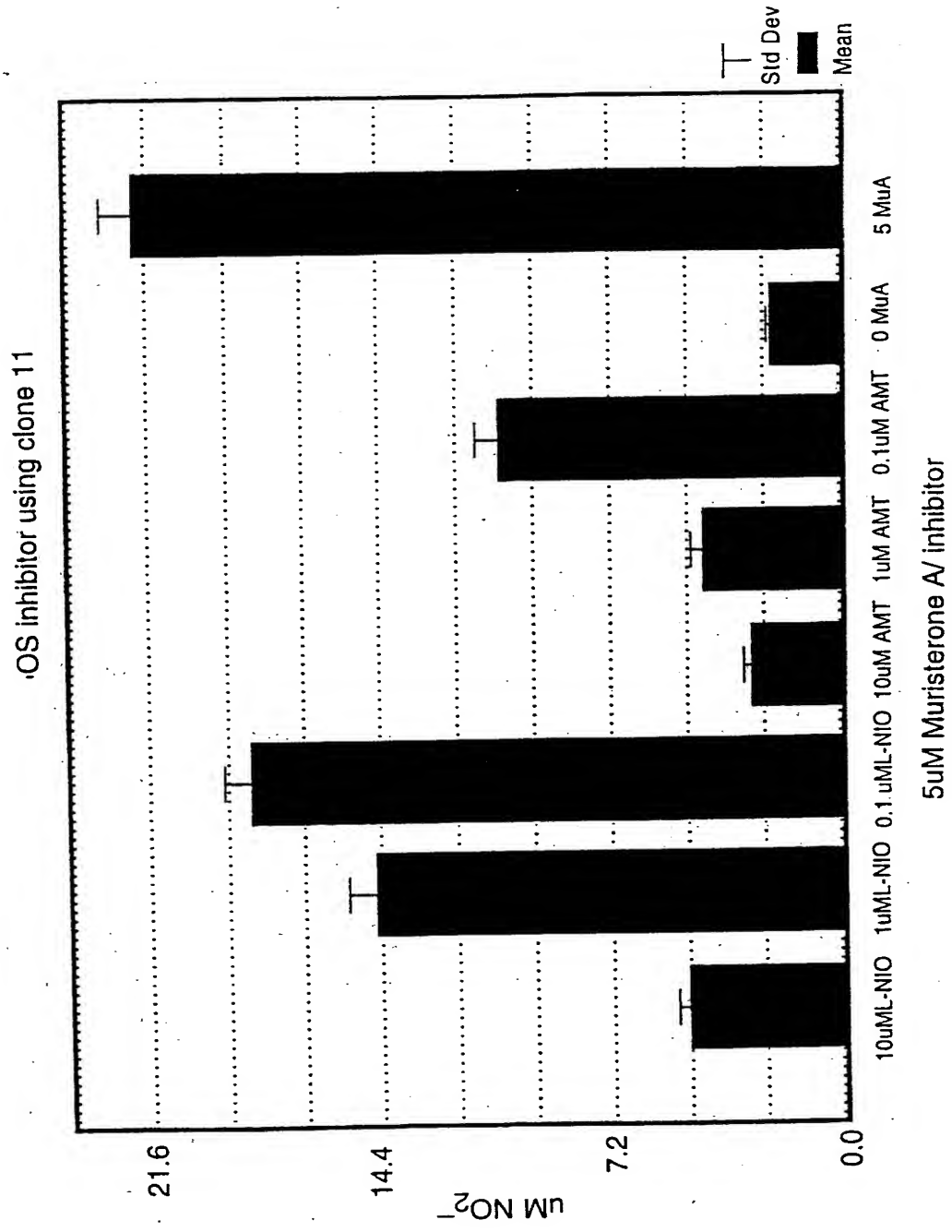

Std Dev
Mean
OS inhibitor using clone 11
uM NO2-
21.6
14.4
7.2
0.0
10uML-NIO
1uML-NIO
0.1uML-NIO
10uM AMT
1uM AMT
0.1uM AMT
0 MuA
5 MuA
5uM Muristerone A/ inhibitor

Figure 4

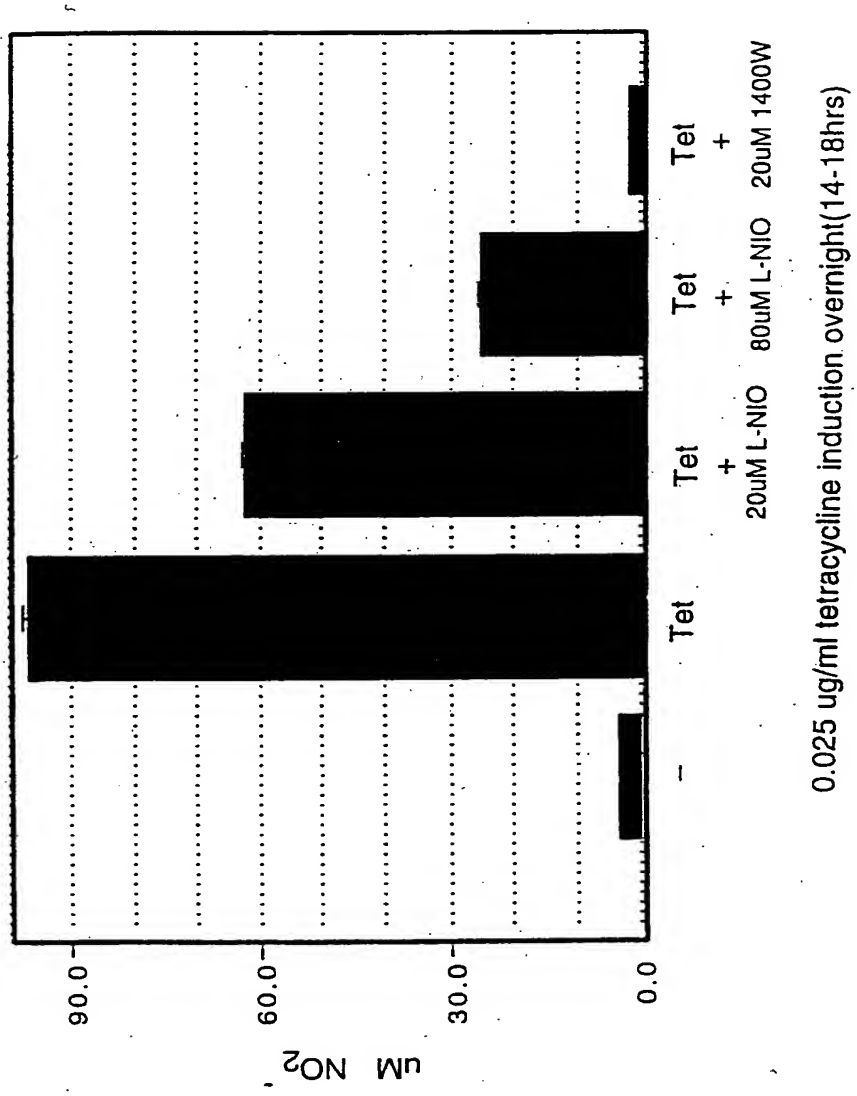

uM NO2
90.0
60.0
30.0
0.0
-
Tet
Tet + 20uM L-NIO
Tet + 80uM L-NIO
Tet + 20uM 1400W
0.025 ug/ml tetracycline induction overnight(14-18hrs)

Figure 5